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A strategy for structuring and reporting a read-across prediction of toxicity ☆

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ABSTRACT

Category formation, grouping and read across methods are broadly applicable in toxicological assessments and may be used to fill data gaps for chemical safety assessment and regulatory decisions. In order to facilitate a transparent and systematic approach to aid regulatory acceptance, a strategy to evaluate chemical category membership, to support the use of read-across predictions that may be used to fill data gaps for regulatory decisions is proposed. There are two major aspects of any read-across exercise, namely assessing similarity and uncertainty. While there can be an over-arching rationale for grouping organic substances based on molecular structure and chemical properties, these similarities alone are generally not sufficient to justify a read-across prediction. Further scientific justification is normally required to justify the chemical grouping, typically including considerations of bioavailability, metabolism and biological/mechanistic plausibility. Sources of uncertainty include a variety of elements which are typically divided into two main issues: the uncertainty associated firstly with the similarity justification and secondly the completeness of the read-across argument. This article focuses on chronic toxicity, whilst acknowledging the approaches are applicable to all endpoints. Templates, developed from work to prepare for the application of new toxicological data to read-across assessment, are presented. These templates act as proposals to assist in assessing similarity in the context of chemistry, toxicokinetics and toxicodynamics as well as to guide the systematic characterisation of uncertainty both in the context of the similarity rationale, the read across data and overall approach and conclusion. Lastly, a workflow for reporting a read-across prediction is suggested.

1. Introduction and problem formulation

Legislative requirements for registration and safety assessment of chemicals have demonstrated the need for a new way of thinking to obtain toxicological information without resorting to animal testing. The grouping of substances allowing read-across of toxicity is a valuable method to obtain such information and potentially has a number of regulatory applications. The underlying philosophy of read-across is that substances which are similar in chemical structure will have similar properties and thereby, have similar toxicokinetic and toxicodynamic properties. Therefore, experimentally-derived toxicological properties from one

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substance, often referred to as the source chemical, can be read across to fill the data gap for a second substance, the target chemical, which has a similar chemical structure and for which a toxicology study may be lacking.

Despite the fact that read-across has been used for several years, a number of challenges remain. For instance, when applying read-across to make a prediction of toxicity, a number of questions arise, for which answers may be difficult to arrive at or to document; including:

(1) Can a robust group of chemicals (often referred to as a chemical category) be formed to include the target chemical?
(2) Is the category formed relevant for the toxicology of the endpoint under assessment?
(3) Are there appropriate toxicology studies of high enough quality for the source chemical(s) to allow a meaningful read-across?
(4) What is the uncertainty and is it acceptable to use the read across prediction to fill the data gap for a specific regulatory purpose?

To begin to address these questions a flexible strategy for developing and reporting a read-across prediction has been created. The strategy focuses on the two main elements of any read-across estimation, namely assessing (1) the similarity between target(s) and source substance(s) and (2) the uncertainties in the read-across process and ultimate prediction. While the standards for accepting a read-across prediction can vary between regulatory agencies, a good basis is the standard required for filling a REACH registration information requirement (EC, 2006). Conceptually, this means, for example, that in the context of a safety assessment for a complete set of results it should be possible to read-across the findings of a 28-/90-day repeated-dose oral rat toxicity study on the source substance(s) to the target substance(s). As such, the aim of the read-across is to provide a prediction(s) that is (more or less) equivalent to the omitted standard animal study and hence be acceptable for regulatory purposes.

The intent of this document is to establish a strategy which may be used to conduct and document read-across predictions for data gap filling. As such, it provides guiding principles for developing read-across predictions for discrete organic compounds. Where possible, emphasis has been placed on undertaking and describing the read-across prediction in the best manner to facilitate regulatory acceptance. This document represents, in part, discussions in and progress made in the European Commission and Cosmetics Europe funded SEURAT-1 Cluster (www.seurat-1.eu). As such, the primary focus of this document is directed towards read-across predictions for chronic toxicity, or improving the possibility to read-across from repeat dose toxicity tests. However, in order to achieve this aim, the document draws upon current expertise and knowledge from other toxicological endpoints and the information, templates and work plans contained herein are generally applicable to all read-across scenarios and endpoints.

In order to facilitate regulatory acceptance, a read-across prediction needs to be justified in all aspects. Briefly, the justification of a read-across prediction needs to be robust, reliable and easily explicable. Key principles of similarity need to be clearly documented and, where possible, supported by scientific literature and data. Sources of uncertainty need to be identified and accommodated; these can typically be divided into two main types: (1) the uncertainty associated with the justification of similarity between the source and target structures, and (2) the uncertainty associated with the application of the particular read-across exercise.

Whilst no consensus has been reached by stakeholders and users, there is growing agreement that when read-across is applied to make predictions to fulfil information requirements, this must be done on an endpoint-by-endpoint basis, i.e. for the particular toxicology study to be predicted. This approach to apply to endpoints individually is due, even when there is an over-arching category hypothesis, to different applicability domains, different source chemicals and/or different Weights-of-Evidence (WoE) which may apply to making predictions for different endpoints. Obviously, there will be occasions where one or more endpoints will be closely related and knowledge may be transferable, thus allowing read-across arguments to build, partially, on each other.

It is generally agreed that the acceptability of a read-across prediction relies on the explanation of the similarity which forms the basis of the read-across, as well as the description of the type and degree of uncertainty associated with the particular read-across. Therefore, it is important to address these two elements in a transparent and consistent manner. The use of templates or work plans facilitates the elucidation of the transparency and consistency in read-across. Existing templates or reporting formats for read-across vary in detail, however, it is generally agreed that they aim to:

(1) Describe the rationale for the similarity between the source and target chemical in a transparent manner.
(2) Document the logic and data leading to the read-across prediction so that, if required, it can subsequently be recreated.
(3) Describe the uncertainties in the prediction; specifically separating the uncertainties in data and definition of similarity from procedural uncertainty.
(4) Clarify the roles of any endpoint specific and/or endpoint non-specific factors affecting the assessment.

2. Background

Read-across is an alternative method for filling data gaps based on an analogue or chemical category approach (van Leeuwen et al., 2009). It is the process of assessing a toxic endpoint of an untested substance (i.e., target chemical) based on the results for the same endpoint for a tested substance (i.e., source chemical) considered to be "similar" in the context of structure, properties and/or activities (Dimitrov and Mekenyan, 2010). It is recognised that forming a chemical category and data gap filling by interpolation within the category, especially for hazard assessments, is not a new concept (OECD, 2014a). However, greater emphasis has now been placed on the resultant read-across prediction due to legislative pressure, especially within Europe, and especially for classification and labelling, and risk assessment. Currently, there is growing interest in several national Governmental regulatory agencies to establish best practices for conducting and evaluating read-across within the context of, and to enable, regulatory decisions. Published exercises and case studies using the OECD QSAR Toolbox (cf. Enoch et al., 2013) have demonstrated that category-based read-across can be used to establish that a substance is associated with potentially hazardous properties. However, it is more difficult to show that a substance is not potentially hazardous. In order to address this issue, the more recent literature has identified some of the challenges which need to be taken into account when preparing a read-across justification (cf. Patlewicz et al., 2013a, 2014); specifically, case studies have described the process to create a read-across prediction increasing the likelihood of regulatory acceptance (cf. Ball et al., 2014).

Much guidance on grouping of chemicals and read-across is already available (ECETOC, 2012; ECHA, 2009, 2011; OECD, 2007, 2011, 2014a) and the key strategic documents have been summarised in Table 1.4 of Cronin (2013a). This is a fast moving field and the formation of chemical categories, or the grouping of molecules, especially to allow for the filling of data gaps by read-across,

has advanced markedly since the start of the 21st Century. Background information on the processes of grouping and read-across has been detailed by Cronin et al. (2013). It is clear that interest in chemical category formation, coupled with read-across for toxicological data gap filling, has grown for a number of reasons (Cronin, 2013a). However, the primary drivers of this expansion are legislation, which has forced the need for non-test methods to assess chemical safety and the willingness of regulatory bodies, although it is cautious, to accept read-across-based submissions in lieu of test results. While there are various advantages and disadvantages to using the category-based read-across approach in toxicology (Patlewicz et al., 2013a,b; Cronin, 2013a), the advantages appear to out-weight the disadvantages. As additional case studies demonstrating the utility and practical application of read-across become available, the advantages will become more prominent and the challenges more readily addressed.

All applications of read-across are context dependent and any read-across adaptation (i.e., the formal process by which a prediction is used for regulatory purposes) is likely to be performed with limited sets of experimental data. Thus, successful adaptations of a read-across are contingent not only on the appropriate selection of the characteristics, measures of similarity and assessment of the uncertainties associated with the prediction, but also on the quality and quantity of the information and data used in the exercise.

Within the applicability domain of a chemical category, read-across can be performed to fill data gaps with a number of approaches which can be summarised into the following four techniques:

(1) one-to-one read-across (i.e., one source substance used to make a prediction for a single target chemical),
(2) many-to-one read-across (i.e., two or more source substances used to make a prediction for a single target chemical),
(3) one-to-many read-across (i.e., one source substance used to make a prediction for two or more target chemicals), or
(4) many-to-many read-across (i.e., two or more source substances used to make predictions for two or more target chemicals).

Techniques 3 and 4 may be considered as being multiple simultaneous applications of techniques 1 and 2, respectively. Given limited data availability, the "one-to-one", or analogue approach, is often the only viable option. Ideally, however, the "many-to-one" or category approach is preferred as it inherently possesses a greater WoE in that each analogue in the category supports the others.

With reference to the above applications (one/many-to-one/many), it is recognised that read-across for toxicity prediction can be qualitative or quantitative in design. A qualitative read-across provides a "yes/no" prediction for an effect; quantitative read-across provides quantitative (i.e., potency) values for an endpoint. When conducting a quantitative read-across exercise, the OECD suggests that there are four main approaches to making the prediction (OECD, 2014a):

(1) reading across from the endpoint value of a similar chemical (e.g., the closest source chemical);
(2) applying a mathematical scale to the trend in available experimental results from two or more chemicals similar to the target chemical (e.g., trend analysis or structure–activity relationships);
(3) processing the endpoint values from two or more source chemicals (e.g., by averaging, by taking the most representative value), or;
(4) when sufficient data allow, taking the most conservative value among the source chemicals within the whole category.

Establishing similarity on an apical endpoint-specific basis is essential to successful category formation and read-across (ECETOC, 2012). Chemical similarity can be considered in a number of ways (Enoch and Roberts, 2013). Critical to the justification of analogue(s) selection for read-across is the explanation of seminal criteria of chemical similarity on which the selection is based. The definition of these criteria is an on-going issue since chemical similarity may be assessed in many ways and, even when assessed objectively, not all measures of chemical similarity are of equal importance and there is no simple similarity scale. In the extreme, each chemical can be considered as its own category; however this is obviously not practical for predictive purposes. In addition, it is accepted that simple measures of chemical similarity (e.g., being a member of a simple organic chemical class, having the same carbon skeleton or same function group) are often not practical for making predictions. Thus, as noted by Enoch and Roberts (2013), in order for any read-across prediction to gain acceptance, it is essential to explain the basis for similarity between the target chemical(s) and source chemical(s) in a robust and reliable manner.

After a read-across exercise is carried out, an assessment is undertaken of whether the case supporting the read-across is sufficient for the prediction to be acceptable. This acceptance is often stated in the form of confidence or certainty. While the acceptance of read-across predictions is often made according to a standard procedure (e.g., an assessment framework), ultimately the evaluator(s) must be convinced of the scientific credibility of the premise of the read-across and the supporting data provided. Therefore, assuming the rationale for similarity is accepted (i.e., the category is robust and membership is assured), final acceptance of the read across prediction is contingent on reducing uncertainty. While uncertainty is related to the quality and quantity of the read across endpoint data (Cronin, 2013b; Péry et al., 2013; Blackburn and Stuard, 2014), there are a number of other factors that influence uncertainty.

2.1. Regulatory context and international efforts to address read-across predictions

In order to understand the context of the development of read-across, it is important to consider how it has been developed and shaped as a data gap filling approach with regard to legislative and regulatory pressure. Globally, a multiplicity of regulatory agencies is applying read-across in their decision making processes. While a number of these agencies are currently focusing efforts on how to best standardise the development and evaluation of read-across predictions, the European CHemical Agency (ECHA), especially through the provisions in Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) is among the better known. Specifically, REACH allows for adaptations to the standard information requirements by means of read-across of a study conducted on a source substance to a target substance (cf. Annex XI in EC, 2006).

The standard ECHA advice to registrants on making and documenting a good-quality read-across/category (ECHA, 2013a,b) refers to the importance of making a clear read-across hypothesis and justification. Non-testing approaches to data gap filling have also garnered much attention at the Organisation for Economic Co-operation and Development (OECD) and among its member countries. Specifically, among the OECD member countries, read-across is used as an alternative method for hazard identification and characterisation in risk assessments; read-across is

especially useful when based on grouping approaches, because not every chemical needs to be tested (OECD, 2014c).

Since the regulatory use of read-across relies on the scientific validity and the robustness of the justification substantiating the prediction for a given endpoint(s), there are a number of issues associated with read-across which may benefit from international discussion on a broader scale. Experiences reported by the OECD members indicate that there is still a lack of agreement on what "chemical similarity" is. Specifically, the OECD has noted the challenge posed by the facts that: (1) a chemical category is defined by a variety of factors, (2) there are no simple similarity scale(s), and (3) similarity can also depend on the endpoint under consideration (OECD, 2014c).

Work at OECD has revealed that similarity hypothesis can be based on a variety of aspects, and definitions, of, chemistry. OECD has also concluded that these methods of assessing similarity are not equal in obtaining a robust chemical category for toxicological read-across. Read-across based on mechanistic similarity (e.g., common chemical interaction with a receptor) is generally considered a better similarity hypothesis than an informatics based similarity metric. However, knowledge of the mode or mechanism of action is not always available, especially for the more complex endpoints such as repeated dose toxicity. Moreover, information on transformation products and the rate of formation of these products is likely to be the key factor in accepting read-across predictions. Thus, information derived from experimental studies, as well as toxicokinetic information and ADME information, will contribute to justify the prediction.

The current view of OECD (OECD, 2014c) is that more experience is needed on how the confidence in the prediction could be enhanced by providing more mechanistic transparency, using experimental data from structural analogues, using data that are supplemented by toxicokinetic and ADME information, and using data that are supplemented by relevant *in vitro* and *in chemico* endpoints (i.e., incorporation of more information to increase the WoE). More specifically, the OECD has emphasised the following as being crucial to the successful application of read-across: (1) the process of how to document the justification for a read-across, (2) consideration of how to perform read-across for more complex endpoints (e.g., repeated dose toxicity), (3) development of approaches and agreement of use of quantitative read-across for hazard characterisation, (4) methods to better take mechanistic considerations into account in grouping chemicals, and (5) approaches to derive WoE conclusions based on results from alternative methods or supplementary information provided.

While the details may vary, it is obvious from all the regulatory requirements and guidance that any general strategy to assess the justification for a read-across prediction must examine whether or not the key principles of similarity are clearly documented and whether the interpretation is supported by scientific justification based on argumentation, literature and data. Development of the similarity rationale, whether for an analogue or a chemical category, must be performed on a case-by-case basis. This case-by-case basis is likely to be influenced by the availability of suitable data to populate the category and be specific to the regulatory endpoint being evaluated (i.e., complex endpoints may intrinsically require greater confidence in the similarity argument and data). Read-across arguments often adopt a multifaceted approach that combines several similarities into a single rationale. This approach, where similarity between the source and target chemicals is demonstrated across multiple parameters, is designed to reduce uncertainty associated with the read-across prediction.

Acceptance of a read-across prediction is often couched in the evaluator's sense of confidence or, more accurately, certainty in the prediction. In the end, high confidence (i.e., low concern about potential error in the prediction) is assigned to a read-across when there is strong proof the prediction is valid (i.e., low uncertainty). This confidence is often gained by identifying and addressing the sources of uncertainty.

Finally, it is recognised that the OECD is currently conducting further work on the hazard assessment of chemicals. Through the Task Force on Hazard Assessment, the OECD is developing Integrated Approaches to Testing and Assessment (IATA). Included in this effort is the examination of grouping approaches and the exchange of experiences among the member countries on new hazard assessment methodologies. A goal of this work is to achieve a harmonised approach to the implementation of IATA, so as to ensure consistency in how information is used in regulatory decision-making and to foster mutual acceptance of assessments (OECD, 2014c). This knowledge and experience will add to the understanding of the process of category formation and use of read-across.

3. Defining the criteria for category membership: establishing similarity

To meet regulatory needs, the read-across hypothesis, or justification for the read-across within a defined chemical category of discrete organic substances, must include a clear definition of the criteria (i.e., chemical similarity) for membership of the category (i.e., a clear definition of the applicability domain). Within the REACH regulation, read-across is founded on the principle of "structural similarity" combined with a scientific justification. Therefore, within the OECD guidance for read-across, the basis for assessing similarity is typically elaborated with the possibility of other considerations (e.g., bioavailability, toxicokinetics/metabolism) to assess analogue similarity (OECD, 2014a). Moreover, a useful tool that might be employed for demonstrating commonality in toxic behaviour is through an adverse outcome pathway concept; this implies assessing similarity "via molecular initiating events", "key intermediate events" and "other relative *in vitro*" information and data (OECD, 2013, 2014b). Clearly, the basis for establishing the applicability domain of a category will depend both on the endpoint and chemical and means of forming a category e.g. a specifically vs. broadly defined fragment. Thus, the questions "Can a chemical category be formed?" and "Is the category toxicologically relevant?" are often addressed concurrently.

Building on six case studies using the information within the OECD QSAR Toolbox (Enoch et al., 2013) and the earlier work of Blackburn et al. (2011) and Wu et al. (2010), it is clear that chemical category membership can be defined by many factors. Table 1 summarises the factors leading to category membership being adequately defined and supported into three elements.

While there can be a starting premise or over-arching rationale for grouping organic substances based on molecular structure and chemical properties, these similarities alone are generally not sufficient to justify a read-across prediction. Typically, further information is required to justify the chemical grouping on the basis of considerations such as bioavailability, reactivity, and metabolism. Similarity in bioavailability is also crucial to confirm where possible. Read-across should be performed where similar bioavailability can be demonstrated. Currently, without experimental data, it is difficult to obtain realistic estimates of bioavailability *in silico*, however progress is being made in areas such as predicting metabolism and clearance rates which combined could provide usable descriptors. For read-across predictions for the less complex endpoints (e.g., acute aquatic toxicity), adding these toxicokinetic similarities is often enough to justify a read-across prediction. However, for the more complex endpoints (e.g., chronic health toxicities), additional measures of similarity are necessary for read-across prediction to be acceptable.

Table 1
Criteria for category membership.

(1) A description of structural and chemical property similarities and differences among the category analogues and how these similarities and differences are linked to the read-across hypothesis
   a. Supported by a data matrix of key structural and chemical properties
(2) A description of toxicokinetics and/or abiotic transformation similarities and differences among the category analogues and how these are linked to the read-across hypothesis
   a. Supported by a data matrix of abiotic and biotic modification properties, including a summary of metabolic pathways and metabolites
(3) A description of the similarity and differences in the bioavailability of the chemical analogues and how these are linked to the read-across hypothesis
(4) A description of biological and toxicological similarities and differences among the category analogues and how these are linked to the read-across hypothesis
   a. Supported by a data matrix of biological and toxicological properties including a summary of toxicological trends within the category

While there is no definitive list of similarities within a group, eleven similarities which are proposed that to have an impact on forming the chemical category for a read-across prediction, are summarised in Table 2. In order to be both transparent and comprehensive, it is suggested to collect similarity data for as many criteria as possible. Whilst molecular structure similarity is a highly pragmatic approach to identify potential source analogues, it is not on its own sufficient to justify read across, and indeed it may not be the most important element.

Data for molecular structure and physico-chemical properties to support grouping hypotheses can be easily obtained *in silico* from software such as the OECD QSAR Toolbox. Using two-dimensional molecular structure, structural data can be organised into groups of atoms representing rings (e.g., benzene or naphthalene), linkers (i.e., atoms in a direct path connecting two ring systems), frameworks (i.e., the combination of ring systems and linkers in a molecule), and side chains (i.e., non-ring, non-linker atoms) (Bemis and Murcko, 1996). These molecular scaffolds provide a basis for assessing similarity. Common constituents include substituents (e.g., the 166 well-characterised, common organic moieties described by Hansch and Leo (1979)) and structural fragments (e.g., the 645 fragments used in the US EPA's the Analog Identification Methodology (AIM)). In addition, physico-chemical and molecular property similarities include properties which are linked to key factors that affect toxicity (e.g., volatility, solubility, reactivity, etc.) (http://www.epa.gov/oppt/sf/tools/aim.htm).

Five types of similarity (Items 3–7 in Table 2) are typically considered to meet the similarity hypotheses for grouping chemicals for read-across based on common toxicokinetics and/or abiotic transformation; these factors largely focus on metabolism which often has significant uncertainty associated with it due to the potential difficulty in obtaining experimental or *in silico* data. Transformation similarities focus on the likelihood of attaining common or similar precursors and/or breakdown products, via physical or biological processes. This includes key abiotic transformations (e.g., hydrolysis, autooxidation) and toxicokinetics (ADME), the same key metabolic pathway(s) or pathway inhibition, activation to same or similar reactive chemical species and degradation to the same or similar chemical species.

For read-across based on common biological/toxicological factors, three types of similarity; toxicophores, mechanistic plausibility and related endpoints, are mostly considered (Table 2), the most important of which is mechanistic plausibility. The AOP construct, an excellent concept for adding mechanistic understanding into the read-across, is one of several means of establishing mechanistic plausibility. In addition, similarity in the biological (preferably *in vivo*) data, such that are available will provided additional evidence for category membership,

In the initial phase of developing a read-across, it is advisable to collect information on similarity and data for as many of the criteria listed in Table 2 as possible. However, it is intuitive that the most critical measurements of similarity are endpoint- and scenario-dependent and hence will require expert judgment and application. In amassing information on similarity (for regulatory applications in particular) it is essential to explain the basis for the similarity between the target chemical(s) and the source chemical(s) in sufficient detail to be able to judge fit for purpose. There are a number of potential regulatory purposes for performing, and uses of, a read-across prediction. The regulatory purposes include: (1) prioritisation and screening, (2) hazard identification (potential), (3) hazard characterisation (potency), and (4) safety assessment (potential/potency and exposure). Thus, in assessing the similarity associated with grouping, it is important to do so in the context of the decision being considered and the scope of the problem. The "context" and "scope" significantly influence a number of issues including the similarity rationale(s) required to form the category and identify analogues.

The regulatory purpose of the read-across often determines the type(s) of similarity required. It is currently accepted (c.f., Cronin et al. (2013)), that there are three broad criteria of similarity: (1) chemistry, (2) transformation, and (3) toxicology. In consideration of prioritisation and screening, hazard identification and safety assessments greater and more detailed information is required

Table 2
Similarities for establishing a toxicological read-across.

(1) Molecular structure similarity including common chemical class and sub-class(es), similar molecular scaffold(s), similar numbers of carbon atoms and common constituents in the form of key substituent(s), structural fragment(s) and extended structural group(s)
(2) Similar physico-chemical and molecular properties, especially those that are linked to key factors that affect bioavailability toxicity (e.g., volatility, solubility, reactivity, etc.)
(3) Similar toxicokinetics
(4) The same key abiotic transformation process (e.g., hydrolysis, autooxidation)
(5) The same key metabolic pathway(s) or pathway inhibition
(6) Biotic and abiotic activation to the same or similar reactive chemical species
(7) Abiotic (e.g. microbial) degradation to the same or similar chemical species
(8) Similar structural alert, or toxicophore, (i.e., structural fragment(s) and extended structural group(s) experimentally demonstrated to be associated with a specific toxic effect that is causally linked with the *in vivo* endpoint which is read across)
(9) Mechanistic plausibility, especially in the form of a common Adverse Outcome Pathway (AOP) based Molecular Initiating Event (MIE) and/or key intermediate event(s) causally linked to the *in vivo* endpoint which is the basis of the read-across
(10) Other data (e.g., *in vitro*, *in chemico*, *in silico*) relevant to the *in vivo* endpoint which are the basis of the read-across
(11) Similarity in *in vivo* toxicological responses within the category

on similarity as described further in Section 4. Therefore, in order to achieve the goal of "fitness for purpose" (i.e., to be both transparent and comprehensive in the justification of a read-across) it is advisable to collect data for as many of these similarity criteria listed in Table 3 as possible. To assist in this process of collecting and assessing information relating to similarity, a template for assessing similarity of analogues and category members for read-across has been proposed and is reported in Appendix A.

This proposed template to collect information to establish similarity includes an overall conclusion regarding the rationale for analogue/category similarity (this is provided as a text box in the Template in Appendix A). The conclusion is intended to summarise all relevant scientific information relating to establishing similarity, in order to clearly justify the analogue(s) selected. The overall rationale for similarity is established by assessing the various criteria for similarity. This is achieved by answering the following questions relating to chemical, transformational and toxicological similarity.

4. Confidence and uncertainty

There is general agreement that increased uncertainty has a strong negative impact on a read-across prediction and often negates the use of the read-across method. For that reason, uncertainties need to be identified and appraised (Cronin et al., 2013; Ball et al., 2014; Blackburn and Stuard, 2014; Patlewicz et al., 2014). However, the concept and definition of uncertainty has been described as ambiguous; it tends to incorporate a variety of methodologies with the aim of meeting different goals (Péry et al., 2013). As a result, a major challenge for the better use of the read-across approach lies in making the concept of uncertainty more understandable and transparent. Currently, determining how much uncertainty is acceptable for a read-across prediction is still largely subjective. It is defined on a case-by-case basis and influenced heavily by the purpose of the prediction, the endpoint assessed, and whether the read-across predicts the presence or absence of toxicity.

To date, the most comprehensive method for gauging uncertainty for read-across, especially for chronic health effects (e.g., repeated dose toxicity), is in the "framework" of Blackburn and Stuard (2014). This is a prescriptive scheme for addressing the various facets of uncertainty as it pertains to read-across. Specifically, it is designed to: (1) increase transparency of the read-across prediction, (2) provide consistency to the exercise, (3) provide a means of examining robustness and consistency among the key facets of similarity, (4) facilitate review and evaluation of the read-across exercise, and (5) help identify where additional data may be helpful, especially in reducing uncertainty. The Blackburn–Stuard framework does not, however, completely remove subjectivity from the process, as expert judgment is still required to categorise uncertainty. In addition, the Blackburn–Stuard framework defines four levels of uncertainty (i.e., low, low to medium, medium and high) and proposes quantitative factors (i.e., 1, 3 and 10, respectively) for addressing the three lesser levels, with the uppermost level of uncertainty being deemed unsuitable for the application of the read-across method. The numerical uncertainty factors serve to build conservatism into the potency prediction and weigh the unknown associated with the prediction. While the framework is new and largely untested, the scheme appears to be good for repeated dose toxicity endpoints where assessment factors can be applied to NOAELs. More quantitative approaches for assessing uncertainty are provided below.

Sources of uncertainty include a variety of elements which are typically divided into two main issues. The first issue is uncertainty associated with similarity justification, and the second is associated with the overall approach and conclusion. With regard to the uncertainty associated with similarity justification, this implies that there are inherent uncertainties associated with the presumption that the results of the *in vivo* study/ies on the source chemical(s) apply (i.e., can be read across) to the target analogue(s). The justification for this presumption is based on two interrelated rationales: (1) that the target and source materials are sufficiently similar to be toxicologically relevant, and (2) that supporting arguments are provided to justify that the differences in chemical structure do not affect the properties relevant to the specific endpoint under consideration.

The assessment of uncertainty associated with similarity justification includes consideration of the information supporting the scientific arguments for similarity and data associated with the chemical, toxicokinetic and toxicodynamic similarity resulting in the toxicity being read across. As stated previously, chemical-based toxicological similarity may be established by responding to the questions posed in Table 3 which may be achieved by following the template presented in Appendix A. Uncertainty associated with the answers to the questions in Table 3 is assessed in a uniform manner and a WoE, indicating consistency in quality and quantification of the data for each feature, assigned (Appendix B, Table B.1).

Among the uncertainties are those brought about by deficiencies in the underlying knowledge and data associated with assessing the essential areas of similarity. Chemical similarity, in itself, may never be enough to justify fully a read-across prediction. While molecular structure and physico-chemical properties play a role in assessing similarity, depending on the toxicological endpoint under consideration, these factors by themselves may not be enough. For example, for chronic health endpoints, two structurally similar chemicals may have significant differences in toxicity. In these cases, toxicokinetic and/or biological similarity may be more important. When such information is lacking, specific studies

Table 3
Criteria to establish similarities for a toxicological read-across.

- What are the chemical identifiers and structure of the target substance(s) and the source analogue(s)? (see Appendix A, Table A.1)
- Define the similarity in the physico-chemical and molecular properties of the target substance(s) and the source analogue(s). (see Appendix A, Table A.2)
- Define the similarity of the key substituents, functional group(s) or extended fragment, generic class of chemicals and sub-class of the target of the target substance(s) and source analogue(s) have? (see Appendix A, Table A.3)
- Identify any structural differences between the target substance and source analogue(s)
- Establish how structural differences may affect toxicity (or otherwise) through similarities, for instance, in *in vivo* data
- Define the similarity in abiotic transformations and/or toxicokinetics between the target substance and source analogue(s) (see Appendix A, Table A.4)
- Define the similarity in potential metabolic products between the target substance and source analogue(s) (see Appendix A, Table A.5)
- Define the similarity in toxicophores or structural alerts for causally-linked toxicological endpoints between the target substance and source analogue(s) (see Appendix A, Table A.6)
- Identify whether the target substance(s) and source analogue(s) have the same mechanistical plausibility and can be linked mechanistically to the same AOP, MIE or KEs (see Appendix A, Table A.7)
- Identify if the target substance(s) and source analogue(s) are linked by other toxicologically relevant data (see Appendix A, Table A.8)

may be necessary to confirm the premise of the similarity justification or, as a minimum, reduce the uncertainty in the similarity to an acceptable level for the intended purpose. Such a confirmation of biological similarity may be obtained from the comparison of toxicological profiles derived from, for instance, non-animal tests. However, in such cases, it may be complex and require expert judgement to select the appropriate *in chemico* method, *in vitro* assay or possibly an *in silico* tool to provide the critical information needed to strengthen a similarity rationale.

The second issue of uncertainty is associated with the completeness of the read-across argument. The molecular nature (e.g., complexity of molecular structure) of the target chemical(s), the nature and complexity of the apical endpoint to be read across, the premise or hypothesis of the read-across, the purpose of the prediction as well as the quality and robustness of the data all can have an impact on uncertainty, its definition and acceptability for read-across (Table 5).

The molecular nature (e.g., complexity of structure) of the target chemical(s) (2nd bullet in Table 5) implies that target chemicals with simple molecular structures (e.g., a hydrocarbon scaffold and one functional group) impart less uncertainty than a more complex molecular structure (e.g., a heteroatom scaffold with multiple structural groups).

In terms of chemistry, the more narrowly defined the applicability domain of the grouping, the greater the confidence can be placed in the group membership and hence, the less the uncertainty. For example, low uncertainty is associated with all category members having the same functional groups and appropriately similar key physico-chemical and molecular properties (e.g., aliphatic aldehydes with C2 to C5).

Relating to the problem and premise of read-across (1st bullet in Table 5), it is intuitive that reading across from many-to-one provides lower uncertainty than reading across from one-to-one, assuming that the standard of the available *in vivo* data of the source substances, and the trends within them, are comparable. Further uncertainty may be associated with the apical endpoint itself, which is to be read across. For some endpoints, chemical mechanism and/or biological modes-of-action are well-established (e.g., mutagenicity). However, for other endpoints (e.g., repeated dose toxicity), the lack of a mechanistic understanding tends to introduce greater uncertainty into the similarity rationale. Mechanistic uncertainty is best assessed within the context of an AOP. It is recognised that knowledge of an AOP evolves and, as such, AOP development represents a continuum from less-to-more complete with increasing quality, quantification and strength of key events (KEs) and key event relationships (KERs) (Tollefsen et al., 2014). Confidence in using an AOP is typically informed by: (1) support for the biological plausibility of KEs, KERs in relationship to the *in vivo* apical outcome under consideration, (2) support for the essentiality of the MIE and other KEs, and (3) empirical data quantifying the KEs and support for the KERs.

As an example, typically, there is more uncertainty with a developmental toxicity endpoint than a genotoxic endpoint. A chemical which can cause DNA or chromosomal damage is deemed a genotoxin. As such, many *in vitro* and *in vivo* tests for genotoxicity have been developed with a range of endpoints that either detect DNA or protein damage or a genotoxicity-related biological consequence; causal linkage between the interaction of a chemical with biomolecules at the molecular level and subsequent *in vitro* and *in vivo* genotoxic effects are well-established (Petkov et al., 2015). The net result is that there are practical methods of integrating *in silico* and *in vitro* results to reduce uncertainty in predicting genotoxicity outcomes of untested chemicals. In contrast, there are a variety of interactions of a chemical with biomolecules which can subsequently lead to adverse developmental effects (Wu et al., 2013). Many of the interactions that underpin developmental toxicity may not be defined in detail and it may not be possible to obtain data for Key Events in the AOP, even for well defined events. Thus, the read-across of developmental toxicity is implicitly associated with greater uncertainty than for well described and "modelled" endpoints. Linked to this concept is the realisation that there are several sources of uncertainty in supporting biological justification. These sources, which are relevant for all systemic endpoints, include: (1) incomplete knowledge of the biological mechanism(s) resulting in toxicity, (2) relevance and completeness of the supporting evidence in the form of scientific information and/or test data, and (3) problems with the test data (e.g., variability in results, lack of understanding what the results mean, etc.). Once the weaknesses or data insufficiencies in the justification are documented, new method evidence can be added to address the shortcomings and reduce the uncertainty.

The read across endpoint(s) is another focal point of the exercise. The type of read-across endpoint affects uncertainty and as more complex endpoints are addressed, there will be a greater WoE required to justify category membership. Simpler endpoints (e.g., acute toxicity) may be readily addressed with fewer lines of evidence supporting the biological justification; often, a single toxicity profiler or small group of *in vitro* tests are sufficient to establish the chemical category or analogue and support the read-across. In contrast, for more complex endpoints, such as chronic health effects which are traditionally assessed by higher level *in vivo* tests (e.g., 28-day repeated dose testing), establishing the category is more difficult. In the case of complex endpoints, analogues are often identified by WoE, looking at consistency in empirical and/or model data across a number of mechanistically relevant endpoints. For example, read across for skin sensitisation may require a WoE call after gauging uncertainty in skin metabolism or abiotic oxidation, as well as chemical reactivity leading to protein binding and dendritic cell activation. In contrast, reading across for oral *in vivo* mutagenicity may require gauging uncertainty in microbial transformation in the gut, metabolic activation in the liver and chemical reactivity leading to DNA-binding and would probably require a lower overall WoE than for chronic toxicity. The depth and breathe of the information and empirical data for these different activities affect the overall level of uncertainty allowed, while still accepting the prediction via the WoE.

The problem and premise of the read-across significantly influence both the similarity rationale required to form an appropriate chemical category and the empirical data of sufficient quality required for the source chemical. Thus, taking the scenarios summarised in Table 4, in Scenario 1 toxicokinetics are less critical to establishing similarity and establishing a source chemical as being of high quality than in Scenario 2. In fact, the absence of toxicokinetic data for Scenario 2 may mean the uncertainty is too great as to prevent the use of read-across without further testing. In addition, a read-across prediction of the absence of an adverse effect carries with it a greater perception of uncertainty. In this case it is not possible to demonstrate with absolute certainty that a target chemical does not elicit a particular *in vivo* adverse effect (Scenario 3), however it may be possible to reduce uncertainty by demonstrating the absence of sub-cellular and cellular responses (i.e., negative results from molecular screening and toxicogenomics). In Scenario 4, one of the key questions to be addressed is whether sub-categorisation is required to reduce the uncertainty associated with the applicability domain of the read-across. The purpose of the prediction also impacts the degree of uncertainty that is acceptable.

While most previous publications discussing read-across have focused on its application in safety assessment, read-across may be used to fill other needs. As noted earlier, there are four regulatory uses for using read-across predictions that apply three basic types of similarity. The purpose of the prediction may determine

Table 4
Summary of the main types of read-across scenario.[a]

1. Chemical similarity of compounds that do not require (or do not undergo) metabolism to exert a potential adverse human health effect (i.e., direct-acting toxicants with a similar mode of toxic action)
2. Chemical similarity involving metabolism and resulting in exposure to the same/similar toxicant (i.e., indirect-acting toxicants with a similar mode of toxic action based on metabolites with the same mechanism of action)
3. Chemical similarity of compounds with low general or no toxicity (i.e., toxicants with no obvious reactive or specific mode of action)
4. Distinguishing chemicals in a structurally similar category with variable toxicities based on Mode of Action hypothesis (i.e., toxicants with high structural similarity but markedly different potency and/or phenotypic profiles)

[a] From Schultz (2014).

Table 5
Proposed factors affecting uncertainty associated with the mechanistic relevance and completeness of the read-across.

(1) The problem and premise of the read-across. What is the level of complexity of the read across endpoint? What is the purpose of the exercise? What is the overarching premise and scenario of the exercise?
(2) Number of source chemicals and their relative applicability domain(s); is it an analogue-or category-based read-across?
(3) Absence/presence of toxicity and relevant mechanisms e.g. whether mechanisms can be defined for non/low toxicity compounds
(4) Quality of the *in vivo* apical endpoint data read across to include technical issues related to the performance (e.g., reliability accuracy, precision, repeatability and reproducibility of the manner in which apical *in vivo* data are generated). Is the data to be read across sufficient to meet the purpose of the exercise?
(5) Consistency in the severity of the apical *in vivo* hazard. Is the potency of the hazard consistent among the source chemicals?
(6) Robustness of the (*in chemico*, *in vitro* and/or other) data sets. How extensive are the relevant events empirically measured or modelled? What is the performance (e.g. in terms of reliability and reproducibility) of methodology for establishing these data?
(7) Concordance of the *in chemico*, *in vitro* and/or other data with regard to the intermediate and apical effects and potency data. What is the temporal and dose–response relationship between mechanistically-relevant endpoints?
(8) The overall Weight-of-Evidence (WoE) supporting the prediction. How many and how large are the mechanistically-related data gaps?

the types of similarity required that can be used, and thus influences uncertainty. Prioritisation and screening may be amenable to prediction based only on information from analogue chemistry. Hazard identification may require information on both chemistry and toxicology similarity. However, hazard quantification for risk assessment will normally needs dosing route and transformation similarity to assess exposure and toxicological similarity; in addition there may be an assessment of mechanistic plausibility, perhaps based on an AOP.

The uncertainty that is associated with the *in vivo* toxicology study/ies on the source chemical(s) is always case-specific. Assessments should focus on any deficiencies in the quality of the toxicology data to be read across, especially as compared to what is expected from current standard test methods. Questions 3–4 in Table 5 are designed to address uncertainty associated with the *in vivo* data being read across (a number of methods are available to ascertain toxicity data quality, with the reader being referred to (Klimisch et al., 1997; Przybylak et al., 2012; Steinmetz et al., 2014; Yang et al., 2013) for further information). Conversely, the final three questions in Table 5 are designed to address uncertainty associated with *in chemico*, *in vitro* or *in silico* data used to strengthen the similarity rationale. Lower uncertainty may also be assigned when empirical and *in silico* measurements of chemical properties are in good agreement.

The qualification of transformation impacts uncertainty, especially with respect to metabolism for the category members without empirical data. For example, low uncertainty is associated when all category members have similar ADME properties. Although there is uncertainty associated with predictions from *in silico* tools, the uncertainty is considered lower when empirical studies (*in vivo* and/or *in vitro*) and model predictions indicate similar metabolism. In addition, information on the purity of compounds being considered and read across must be included as this may affect the certainty.

The uncertainty associated with a read-across prediction is impacted by several additional features, especially those associated with the completeness and application of the read-across procedure; this knowledge is typically summarised in an overall assessment of the WoE. In assessing the uncertainties associated with a particular read-across, it is important to put in context both the problem and premise of the read-across. A statement of the problem includes noting the target chemical(s), the apical endpoint to be read across and the purpose of the prediction. Stating the target chemical(s) is critical, as it is one of the focal points of the exercise.

Scaling uncertainty is a formidable challenge (Péry et al., 2013; Blackburn and Stuard, 2014). While there is much agreement on what the essential issues of the read-across are that need to be considered in assessing uncertainty, there is less agreement on what approach to use. At least three approaches could be applied: (1) a sliding scale, which can be tailored to the particulars of the read-across (i.e., problem and premise), (2) a weighted scale, where some issues or their related narrative and/or question(s) used to frame the issue are weighed more than others, and (3) pre-defined divisions, where all issues or their related narrative and/or questions are assigned a value in a parallel fashion. The first two approaches, while interesting academic exercises are likely to be too complex to be practical. Thus, the third approach, the pre-defined divisions approach, is the most likely to be used. Within the latter approach, there is variability in the number of divisions employed. A dichotomous decision scheme (i.e., accept or reject) does not provide any refinement to the assessment; whereas, a multi-divisional scheme will provide the opportunity to add confidence statements into the assessment (e.g., low. medium, high). A five-division scheme (or larger) may offer too much subjectivity in assigning the division. The four-division scheme (i.e., low, low to moderate, moderate and high) described by Blackburn and Stuard (2014), appears to provide a balance between a high number of possible divisions and reduced subjectivity in assigning the final division. The Blackburn and Stuard scheme provides three divisions of uncertainty where the prediction may potentially be usable; with the fourth division indicating high uncertainty such that the read-across method is unfit for data gap filling. The "characteristics by uncertainty" for the low and low-to-medium divisions are much the same, with latter divisions including a WoE evaluation. Initially, read-across case studies are likely to involve extremely-well studied categories and analogues which fit the low uncertainty division of Blackburn and Stuard

(2014). However, in the future, the more common read-across predictions, especially for chronic health effects, should include a WoE evaluation.

Uncertainty factors are used to build conservatism into assessments and address the unknown associated with a prediction. Converting uncertainty "divisions" (as reported by Blackburn and Stuard, 2014) to numerical uncertainty factors provides another challenge. Excluding the "high" uncertainty division, since reaching this level of uncertainty precludes using read-across to fill a data gap, one is left with assigning three uncertainty factors. There are a variety of numerical scales (e.g., 1-2-3; 1-10-100; 1-3-10; 1-5-10) which may be employed to cover a three-division scheme. A 1-2-3 method provides insufficient differentiation of uncertainty; conversely, a 1-10-100 provides too much differentiation. The 1-3-10 method proposed by Blackburn and Stuard (2014) remains a pragmatic and usable solution and is recommended for use at this time. However, as case studies become available, especially for those where the read-across is less conclusive (i.e., low-moderate or moderate), further evidence may become available to evaluate this proposal more fully, for example to explore the difference in employing a 1-3-10 versus a 1-5-10 quantification method.

Table 6 summarises the main similarities that need to be considered when assessing a read-across justification, along with how they may be related to specific levels of uncertainty. Table 6 also demonstrates the value of including novel toxicological data to read-across predictions with the aim of decreasing uncertainty. It is likely that uncertainty associated with core structure and functional groups, as well as physicochemical and molecular properties, can be assessed relatively easily. However, because of information gaps, it is likely that uncertainty associated with comparable toxicokinetics and similar mechanistic and toxicological properties, especially for chronic health endpoints, will be more difficult to assess.

Consideration of all the evidence (e.g., the uncertainties defined in queries such as Table 5, supporting data and information etc.) provides the basis for the WoE. It is not only the quantity and quality of evidence that affects WoE but also consistency across all aspects of the information/data used to support the similarity rational and prediction. For example, whilst relative uncertainties may be the same, it is intuitive that reading across from many-to-one with consistent phenotypic expressions of toxicity provides a greater WoE than reading across from many-to-one with varied phenotypic expressions of toxicity. This has particular implications in Scenario 4 of Table 4 where multiple mechanisms of action may be present. In terms of chemistry, a greater WoE is assigned when empirical and *in silico* estimates of chemical properties are in good agreement with measured values. In a similar fashion, the WoE is considered higher when empirical studies of metabolism (*in vivo* and/or *in vitro*) and model predictions indicate similar metabolites. Mechanistic plausibility can be more difficult to consider, however consistent empirical data for the target chemical and, where possible, the target chemical and the source chemical(s) for the MIE and/or other KEs strengthen the WoE. Similar arguments can be made for other relevant, *in vivo*, *in vitro* and *ex vivo* endpoints. Concordance across other endpoints (where data exist) is also a relevant consideration. For example, acute oral LD50 data are not part of the mechanistic understanding for oral repeated dose toxicity but having a consistent trend in empirical data among category members may improve the overall WoE.

A template has been provided to identify and assess uncertainty in a comprehensive and transparent manner. The template is available in Appendix B and it is recommended for use to assess the uncertainty associated with each similarity parameter used in the read-across and to summarise these finding in a statement of uncertainty. The first aim of the template was to identify the factors of the read-across that contribute to uncertainty in the prediction. These include uncertainty associated with the scientific justification of the similarity that defines the applicability domain of the category or source and target analogue, as well as the uncertainty associated with the read-across. The second aim was to define levels of uncertainty and propose quantitative factors for addressing each level.

Table B.1 of the template in Appendix B lists and describes the key issues of chemical, transformation/toxicokinetic and toxicological similarity proposed to assess data uncertainty and WoE (see Tables A.1–A.8). The comment column is not intended to be all inclusive but rather give an indication of the type information that may be included. Table B.2 of the template in Appendix B provides the capability to assess the issues raised in Table 5 above. The aim was to assess the non-similarity-based uncertainty associated with the read-across. The first item in Table 5 focuses on the particular read-across problem being addressed. The second to fourth items address the *in vivo* data relevant to the read-across. Items five and six relate to the mechanistically-related *in chemico*, *in vitro* and "new methods" data. Item seven addresses the overall WoE. While a ranking (i.e., low medium or high) is assigned to each item, the comment section is considered to be more significant and hence of greater value. The overall ranking (low, moderate, high) and a summary of the uncertainty associated with the definition of the similarity of analogues or category members, as reported at the end of the relevant tables, is presented in a text box in Appendix B.

Table 6
Proposed key similarities relating to toxicological read-across and criteria for assessing uncertainty (adapted from considerations in Blackburn and Stuard (2014)).

	Low uncertainty	Low-to-moderate uncertainty	Moderate uncertainty	High uncertainty
Core structural similarity i.e., functional groups, extended fragments (especially those associated with chemical reactivity or its modification)	Highly similar	Highly similar	Similar	Differences in core structure and functional groups
Physico-chemical and molecular properties	Highly similar	Similar, having a consistent trend within values	Minor differences in values	Major differences in values
Abiotic transformation and/or toxicokinetics, especially metabolism e.g., leading to a common metabolite	Evidence demonstrating comparability in abiotic transformation and/or toxicokinetics and the same metabolites, similar bioavailability	Evidence demonstrating comparability in abiotic transformation and/or toxicokinetics and the same metabolites, similar bioavailability	No evidence that abiotic transformation and/or toxicokinetics, especially metabolism are dissimilar	Differences in abiotic transformation and/or toxicokinetics, especially in metabolism
Mechanism of action and toxicological properties	Evidence demonstrating comparability in mechanism supported by an AOP	Evidence demonstrating comparability in mechanism, possibly supported by an AOP	No evidence that mechanisms of action are dissimilar	Differences in mechanism of action and/or toxicological properties

5. Workflow for reporting a read-across prediction

Existing workflows for reporting read-across predictions vary in detail, however the general purpose is to: (1) describe the similarity rationale of the read-across in a transparent manner, (2) document the logic and data leading to the read-across prediction so it can be recreated, (3) describe and address the uncertainties, and (4) clarify the roles of any endpoint specific and/or endpoint non-specific factors affecting the assessment.

In order to assist with developing a workflow for reporting, the combined process of chemical category formation and toxicological read-across prediction can be sub-divided into distinct and definable activities. Cronin (2013a) identified six such procedures associated with development of a read-across prediction including: (1) the identification of the effect and/or endpoint to be predicted by read-across and the "target" chemical(s), (2) the identification the source chemical(s) and other chemicals "similar" to the target, (3) obtaining toxicity data for the category members identified in 1 and 2, (4) definition of the chemical category, (5) making the prediction of toxicity by read-across, and (6) fully documenting the prediction.

More recently, the OECD has provided reporting formats for analogue and chemical category approaches (OECD, 2014a). The documentation of read-across predictions, which are largely based on process of using the OECD QSAR Toolbox, includes a number of steps:

(1) Formulate the problem (i.e., understand the assessment strategy and identify the critical data needs).
(2) Curate chemical structure of the target compound(s) and other category members.
(3) Profile the target compound(s) and other category members.
(4) Develop the similarity rationale for the read-across prediction.
(5) Establish the category selection criteria and search for potential source analogues or category members.
(6) Gather data for the category members and construction of data matrix.
(7) Assess the adequacy and uncertainty associated with the read-across.
(8) Apply read-across to fill the data gap.
(9) Document the analogue/category and read-across prediction.

A workflow proposed for reporting a read-across prediction is presented in Appendix C. This builds on the earlier efforts and reflects the essential points described in this paper to address similarity, the data and to justify the validity of the prediction.

6. Discussion

A significant proportion of REACH registration dossiers include a read-across prediction intended to fill information requirements for higher-tier toxicological studies. In fact, 75% of registration dossiers include read-across or categorisation reasoning (ECHA, 2014) by the registrant.

Improvements in methodology to perform and report read-across prediction require an understanding of the process, specifically around the concept of similarity with regard to two or more chemicals. Berggren et al. (2015) noted that in considering chemical similarities there are different aspects that must be assessed to make the read-across prediction scientifically justified. These similarities include aspects of chemical stability, the possible formation of toxic metabolites, different active functional groups that might lead to similar or dissimilar behaviours, possible routes of exposure and concentrations at the target tissue, biotransformation (prior to reaching, or at, the target organ), or observable trends with or without a mechanistic explanation. To improve and standardise the development and reporting of a read-across prediction, it is, therefore, useful to identify different scenarios by which a read-across prediction may develop. While this is possible to do in several ways, the toxicokinetic fate of the substance, such as whether the compound itself would be available in the target organ or whether it would be its metabolites or reaction products leading to adverse effect, is a critical factor, especially for chronic health effects (Berggren et al., 2015). In addition, Berggren et al. (2015) noted that the toxicodynamic behaviour of the substance and compared similarities of chemicals based on their assumed mechanism of action, including lack of biological activity, is critical to establish a read-across justification.

Category-based read-across adaptations begin with the definition of a chemical category (i.e., establishment of the category's applicability domain). This definition is assumed to be related to the toxicological property to be read across, which results from a trend observed when the property to be read across is plotted against another property that is known for all members of the category (i.e., an indication of toxicological relevance). Read-across to a target substance is deemed possible when the target substance is an unambiguous member of the category and there are one or more measured property(ies) to be read across for other members of the category. Therefore, a category-approach read-across is based on grouping and may rely on one or more observed trends. Category-approach read-across also covers cases where substances belonging to a well-defined category all show the same type and value for the toxicological property to be read across or do not show an effect at all (i.e., a 'low-toxicity' read-across case).

While there is no consensus, there appear to be four most likely scenarios where chemical category formation and subsequent read-across may be used to fill a data gap, especially for repeated dose toxicity. Scenarios for read-across in general are described in Table 4, more specific scenarios for chronic endpoints are given in Table 7.

It is important to remember that defining the criteria for category membership for a particular scenario of chemical category

Table 7
The most likely scenarios for a chronic toxicity endpoint read-across.

(1) A 'low-toxicity' or negative read-across prediction; the category members have structural and chemical similarities, toxicokinetics are simple and based on well-documented or easily predicted (from related chemicals) pathways that lead to rapid degradation and/or elimination and/or generation of non-toxic metabolites and there is no obvious chemical reactivity or bioactivity or specific mode-of-action (i.e., members elicit generic effects but only at high concentrations)
(2) A 'toxicity' or positive read-across prediction; the category members are direct-acting toxicants (i.e., no transformation or transformation does not drive the toxicity) with similar chemical mechanism-of-action and mode-of-toxic action (i.e., members elicit specific effects at similar internal concentrations or according to an established structural-related trend) leading to the same read across effect
(3) A 'toxicity' or positive read-across prediction; the category members are indirect-acting toxicants (i.e., transformation is the driver of toxicity), where the definitive toxicants has the same chemical mechanism-of-action and elicits the same mode-of-toxic action leading to the same read across effect
(4) A 'toxicity' or positive read-across prediction; the category members are structurally and chemically highly similar and initially considered similar in bioactivity. Subsequently, new methods data reveal dissimilarity in bioactivity, often due to the inhibition of a degradative metabolic pathway. Thus, to obtain the appropriate read across endpoint effect (e.g., target organ and disease) requires sub-categorisation

formation and read-across is only the beginning of the exercise. Improvement in the confidence of a read-across prediction can be made by added value in the form of increased WoE. This added value may come from suggestions of how targeted testing and "new-approach" data, especially when applied using the logic of the Safety Evaluation Ultimately Replacing Animal Testing (SEURAT) conceptual framework (White and Knight, 2013), may be used to improve the read-across justification. The increase in justification will be especially true if targeted testing focuses on the weak steps of the read-across argument. In other words, an understanding of how targeted testing may reduce uncertainty is available, for instance as stated in Table 6. The improvement of the robustness of the read-across predictions, when further evidence is added can, in principle, be examined by various means before and after the addition of further evidence.

The intention of this manuscript was to report progress in the development of proposed templates and workflows for recording and evaluating traditional *in vivo* toxicology data, as well as alternative methods (e.g., *in chemico*, *in vitro*) data. Additionally, the intent was to suggest means to standardise the evaluation of similarity and uncertainty so as to enhance the robustness of the read-across prediction and thereby make it more likely to gain regulatory acceptance.

Since there are various over-arching scenarios for category formation and read-across, it is critical to not only state the target chemical and its missing endpoint value but also the hypothesis and assumptions on which the read-across is based. A category/analogue hypothesis typically makes references to several similarity rationales which delineate category membership. For example, for a read-across adaptation of Scenario 3 noted in Table 7 it may be possibly to report:

- Members of chemical category **A** are indirect-acting toxicants of n_1 to n_2 carbon atoms in size with a molecular scaffolding of **B** and the primary functional group **C**.
- Category members elicit a similar chemical mechanism-of-action (e.g., electrophilic reactivity via mechanism **D**), where metabolism via pathway **E** is the primary factor driving the reactivity leading to oral repeated dose toxicity with symptoms/endpoints **F**.
- Category members show rapid and complete absorption from the gut, as the parent compound with first past through oxidative metabolism in the liver to the corresponding electrophile with mechanism **D**. Subsequently, the electrophile elicits the *in vitro* outcome **G** at the cellular level leading to the *in vivo* outcome **F**.
- Category members have similar volatility, bioavailability and oral uptake.
- Reading repeated dose toxic outcome **F** for the source chemical **X** across to the target chemical **Y** is supported by information and data on **A**, **B**, **C**, **D**, **E** and **G**.

Along the same theme, assessments of uncertainty may reveal there are no deficiencies in the quality of the toxicology data to be read across (**F**), especially as compared to what is expected from current standard test methods. However, assessment of uncertainty associated with similarity justification reveals metabolism via pathway **E** to be the weak step of the read-across argument. New methods data following targeted testing may reduce the uncertainty by strengthening this step in the similarity argument.

Conflict of interest

The authors report no conflicts of interest.

Transparency Document

The Transparency document associated with this article can be found in the online version.

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Appendix A. Template for reporting data for assessing similarity of analogues and category members for read-across

In Table A.1, the substance identification information, 2D structure and molecular formula data for the target substance(s) and proposed source analogue(s) are presented for comparison. The purpose of this information is to provide, in a transparent manner, a preliminary basis for assessing similarity.

In Table A.2, selected physico-chemical and molecular property data for the target substance(s) and proposed source analogue(s) are presented for comparison. The purpose of this information is to provide, in a transparent manner, the chemical property basis for assessing similarity. These data may assist in defining the boundaries of the applicability domain of the category, especially in regards to *in vivo* (bioavailability) and *in vitro* (solubility) toxicity.

In Table A.3, substituents, functional groups and extended structural fragments as well as chemical class data for the target substance(s) and proposed source analogue(s) are presented for comparison. The purpose of this information is to provide, in a transparent manner, the chemical structure sub-fragments and chemical class data for assessing similarity. These data may assist

Table A.1
Comparison of substance identification, structure and chemical classifications.

	Target Substance	Analogue 1	Analogue n
Name			
CAS No:			
SMILES			
2D Structure			
Molecular Formula:			

Table A.2
Comparison of physico-chemical and molecular properties.[1]

	Target Substance	Analogue 1	Analogue n
Name			
Molecular Weight:			
Log Kow			
Vapor Pressure			
Density			
Melting Point			
Water Solubility			
	Target Substance	**Analogue 1**	**Analogue n**
Boiling Point			
pKa			

[1] Value typically derived from EPISuite v4.0.

Table A.3
Comparison of substituents, functional groups, and extended structural fragments.

	Target Substance	Analogue 1	Analogue 2
Name			
Key Substituent(s)			
Functional Group(s)			
Extended Fragment(s)			
Chemical Class:			
Chemical Sub-Class:			
Chemical Sub-Class:			

Table A.4
Comparison of abiotic transformation and toxicokinetics.

	Target Substance	Analogue 1	Analogue 2
Name			
	Target Substance	**Analogue 1**	**Analogue 2**
Abiotic Transformation			
Toxicokinetics			

in defining the boundaries of the applicability domain of the category.

In Table A.4, transformation information and data for the target substance(s) and proposed source analogue(s) are presented for comparison. The purpose of this information is to provide, in a transparent manner, assessing similarity in abiotic transformation and/or similarity in the absorption, distribution, metabolism and elimination information.

In Table A.5, the predictions of potential metabolites derived from *in silico* tools data for the target substance(s) and proposed source analogue(s) are presented for comparison. A number of software platforms provide *in silico* predictions of metabolism. These are typically based on simulations run on the parent compound and initial metabolites using well-studied reactions, such as oxidation. Files with name and structure of metabolites should be included for the sake of transparency.

In Table A.6, any toxicophore (i.e., toxic endpoint-specific structural alerts) data for the target substance(s) and proposed source analogue(s) are presented for comparison. A number of software platforms provide *in silico* predictions based on the presence of toxicophores (e.g., OECD QSAR Toolbox, Derek Nexus). The purpose of this information is to provide, in a transparent manner, any chemical structure sub-fragments linked to any relevant biological endpoint for assessing similarity.

In Table A.7, any mechanistic plausibility data including AOP-related, MIE, KEs, KERs or other mechanistically-relevant endpoints for the target substance(s) and proposed source analogue(s) are presented for comparison. With few exceptions (e.g., skin sensitisation), there are currently a limited number of endpoints for which AOPs, MIEs and KEs test methods and data have been formally developed and causally-linked, especially in the form of a KER. However, in the future, these pieces of information will become more and more available.

In Table A.8, any other toxicologically relevant data for the target substance(s) and proposed source analogue(s) are presented for comparison. In some cases, there is relevant data from other sources (e.g., alternative species) which can assist in establishing mechanistic similarity.

Table A.5
Comparison of potential metabolic products.

	Target Substance	Analogue 1	Analogue 2
Name			
Liver metabolism simulator e.g. OECD QSAR Toolbox			
Other software e.g. MetaPrint2D-React software			
Further software for prediction of metabolites			

Table A.6
Comparison toxicophores.

	Target Substance	Analogue 1	Analogue 2
Name			
	Target Substance	**Analogue 1**	**Analogue 2**
Toxicophores			

Table A.7
Comparison of mechanistic plausibility and AOP-related event data.

	Target Substance	Analogue 1	Analogue 2
Name			
Mechanistic Plausibility			
Adverse Outcome Pathway or Mode of Toxic Action:			
Molecular Initiating Event:			
Key Event 1 etc.:			
Key Event Relationship 1 etc.:			
Other Mechanistically-Relevant Events			

Table A.8
Comparison of other toxicologically relevant *in vivo*, *in vitro* and *ex vivo* data.

	Target Substance	Analogue 1	Analogue 2
Name			
Endpoint:			
Endpoint:			

Appendix B. Template for assessing uncertainty for read-across

See Appendix Tables B1 and B2.

Summary of uncertainty

Example: Overall, the uncertainty in similarity of the analogues or category member is low. The key features (i.e., A and B) relevant for toxicity are common within the category. There are only minor differences among the analogues or category members with respect to physicochemical properties. Analogues or category members are considered chemically similar (i.e., C). Analogues or category members are judged to follow the same or similar metabolism. Analogues or category members exhibit a similar toxicological profile (i.e., D and E) with respect to the endpoint in question. It is concluded that the structural difference between analogues, hydrocarbon chain length, has no significant impact on the toxicity being read across.

Appendix C. Work flow for reporting a read-across prediction

1. Statement target substance(s) and the regulatory endpoint(s) that is to be read across

The specific data gap to be filled by the prediction needs to be clearly defined by listing the chemical(s) and toxicity endpoint(s) (i.e., property(s)) for which the read-across prediction is proposed.

Table B.1
Data uncertainty and Weight-of-Evidence associated with the fundamentals of chemical, transformation/toxicokinetic and toxicological similarity.

Similarity Parameter	Data Uncertainty [a] (empirical, modelled) (low, medium, high)	Strength of Evidence [b] (low, medium, high)	Comment
Substance Identification, Structure and Chemical Classifications			**Example:** All category members have CAS numbers, similar 2D structure and belong to the same chemical class/subclass.
Physio-Chemical & Molecular Properties	Empirical: Modelled:		**Example:** All category members are appropriately similar with respect to key physicochemical and molecular properties. There is a high degree of consistency between measured and model estimated values.
Substituents, Functional Groups, & Extended Structural Fragments			**Example:** Substituents, functional groups and extended structural fragments are consistent across all category members.
Transformation/ Toxicokinetics and Metabolic Similarity	Empirical: *In vivo*: *In vivo*: Simulated:		**Example:** Based on *in vivo* and *in vitro* data for multiple category members, there is evidence for similar toxicokinetics and metabolic pathways. Comparison of results from empirical studies and model predictions indicate similar metabolism among all category members.
Potential Metabolic Products			**Example:** Based on *in silico* metabolic simulations, potential metabolic products are similar among all category members.
Toxicophores /Mechanistic alerts			**Example:** Based on *in silico* profilers, all category members contain the same toxicophores.
Mechanistic plausibility and AOP-Related Events			**Example:** Although no AOP is currently available for the hypothesised toxicity pathway, many category members have been tested for what is generally accepted as a mechanistically-relevant event leading to the *in vivo* apical outcome of interest (*a citation could be provided*).
other relevant, *in vivo*, *in vitro* and *ex vivo* endpoints			**Example:** Although not part of the hypothesised toxicity pathway, many category members have been tested for rodent acute oral toxicity and there is general agreement among the reported LC50 values.
Overall uncertainty in similarity of category members: (Low, Moderate, High) Summary: Key features of chemistry are similar within the category. Key features of transformation toxicokinetics and metabolism are common within the category. Category members are considered mechanistically similar. Category members exhibit a similar toxicological profile with respect to *in vivo* toxicity.			

[a] Uncertainty associated with underlying information/data used in the exercise.
[b] Consistency within the information/data used to support the similarity rational and prediction.

2. Description of the analogues or members of the category

2.1. Premise

A premise for the basis of the analogue or category needs to be presented. This hypothesis should note the relational chemical, toxicokinetic and biological/toxicological features (i.e., structural similarities) which are deemed to be collectively relevant to the endpoint(s) being read across and common to target and source substance or all members of the category.

2.2. Justification

The analogue or category should be justified based on available experimental data, especially for the source substance(s). This is a description of the experimental toxicological data for the analogues or category members, presented in a narrative fashion. Typically, this justification will include endpoint-related mammalian toxicity data via appropriate exposure schemes, toxicokinetic and transformation information, as well as relevant *in vitro* data and structure–activity relationships. These data should

Table B.2
Template for assessing uncertainty associated with mechanistic relevance and completeness of the read-across.

Factor	Uncertainty (low, medium, high)	Comment
The problem and premise of the read-across		**Example:** The endpoint to be read across, developmental toxicity, for the category of branched carboxylic acids is well-studied and well-understood, The scenario of the read-across hinges on the inhibition of beta-oxidation of the acid and the subsequent build up of acid in the embryo leading to histone deacetylase inhibitors, increased cell adhesion and concomitant reduced cell motility, prevention of convergent extension during ontogenetic development.
In vivo data read across		
Number of analogues in the source set		**Example:** There are 3 suitable category members with *in vivo* apical endpoint data usable for read-across.
Quality of the *in vivo* apical endpoint data read across		**Example:** High quality empirical data from standard test guidelines for the stated regulatory endpoint exists for 1 category member. Similar non-standard test data of lower quality exists for 2 other category members. All these data are consistent in regards to qualitative description of effects and, where available, similar in quantification.
Severity of the apical *in vivo* hazard		**Example:** Potency data for the *in vivo* apical endpoint (25 mg/kg/day) is limited to a single source substance.
Evidence to biological argument for RA		
Robustness of analogue data set		**Example:** The available data from *in silico*, *in chemico* and *in vitro* studies for the category members were judged to be reliable and conducted under the appropriate conditions.
Concordance with regard to the intermediate and apical effects and potency data		**Example:** There is good agreement between the sequences of biochemical and physiological events leading to the *in vivo* apical outcome. There is consistency and high specificity for the association between the toxicophore and the structural domain of the category. There is general agreement among the dose-response relationships of the tested category members for mechanistically-relevant event(s) which may be assessed *in vitro*.
Weight of Evidence		**Example:** Overall the available information is generally consistent with the stated hypothesis. The sharp structural limitations of the category and narrow range of chemical properties strengthens the WoE. While the toxicokinetics data is limited, the lack of inconsistencies adds to the WoE. While the source substances data is limited, the fact that there is consistent relevant *in vitro* data for 50% of the category members, including the target chemical, strengthens the WoE.
Overall uncertainty of the read across: (Low, Medium, High) Uncertainty associated with the read-across is judged to be low.		

demonstrate that the quality and quantity of *in vivo* data to be read across is sufficient to proceed with the exercise. Moreover, these data should be summarised to show the robustness of the read-across and include any indication of data trend(s) within the category for the different endpoints noted.

2.3. Applicability domain

In a category approach, the applicability domain of the category is described by inclusion and/or exclusion rules that identify the extent of values for category members within which reliable predictions can be made. Examples of this are the range of 1-octanol/water partition coefficients values, functional groups or carbon chain lengths within which the category is appropriate.

2.4. Analogues or category members

Analogues or all members of the category, including target(s) and source substance(s), incorporated in the read-across exercise need to be described in a comprehensive fashion that takes into account unique substance identifiers such as, names, chemical structures and CAS numbers.

2.5. Purity/impurities

A purity/impurity profile for each analogue listed in 2.4 needs to be catalogued. The potential impact of impurities on the endpoint(s) being considered in the adaptation should be identified.

3. Data matrices for assessing similarity

Appendix A presented the template for assessing similarity. These data matrices are the central part of the workflow. They are likely to be the first items examined in any assessed. Data should be reported clearly, logically and unambiguously. The key study results should be noted and referenced. The distinction between experimentally measured and model-derived data should be noted.

4. Statement of uncertainty

Appendix B presented the template for assessing uncertainty. This section concludes with a narrative summary of the uncertainty. Particular consideration needs to be given to pointing out what are considered to be the weak steps of the read-across argument; why they are considered weak and how they impact the uncertainty of the read-across prediction.

5. Statement of the conclusions

Lastly, an overall concluding statement is made with regard to the category and the read across prediction relevant to the regulatory decision (e.g., hazard identification, classification and labelling, risk assessment, etc.) being considered. This should include making the prediction of toxicity by read-across and fully documenting the prediction to include clarifying the roles of any endpoint specific and/or endpoint non-specific factors affecting the prediction.

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